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SHORT COMMUNICATION

Circulating miRNA-375 levels are increased in autoantibodies-positive first-degree relatives of type 1 diabetes patients

L. Bertoccini[1] · F. Sentinelli[1] · M. Incani[2] · D. Bailetti[1] · F. A. Cimini[1] · I. Barchetta[1] · A. Lenzi[1] · M. G. Cavallo[1] · E. Cossu[2] · M. G. Baroni[1]

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Abbreviations

Aabs	Autoantibodies
AUC	Area under the curve
Ct	Cycle threshold
FDR	First-degree relatives
GADA	Acid decarboxylase autoantibodies
IA2	Tyrosine phosphatase autoantibodies
IAA	Insulin autoantibodies
IGR	Impaired glucose regulation
miRNAs	MicroRNAs
ROC	Receiver operating characteristic
T1D	Type 1 diabetes
ZnT8A	Zinc transporter 8 autoantobodies

Introduction

Growing evidence suggests that microRNAs (miRNAs) play a key role in immune system functions as well as in beta-cell metabolism, proliferation, and apoptosis, all processes involved in the pathogenesis of type 1 diabetes (T1D) [1].

MicroRNAs are a class of small noncoding RNAs of 19–22 nucleotides, which act as negative regulators of gene expression by partially pairing to the 3′ or 5′ untranslated regions of their target mRNAs [2]. Recent studies have demonstrated that miRNAs are detected not only inside cells but also in extracellular fluids and body secretions [2]. MiRNAs are secreted by cells via exosomes, and travel in the circulation attached to high-density lipoprotein particles, where cells can take them up through receptor-mediated endocytosis [3]. Blood miRNA levels have been proposed as a new class of biomarkers for diagnosis and prognosis of several diseases, including T1D, and also as new targets for treatments and interventions [4].

Although dysregulated miRNA profiles have been identified in T1D patients, results are inconclusive. In the context of diabetes, particular attention has been focused on miR-375, which has been studied most extensively as a putative biomarker of beta-cell death [4]. Relative expression of miR-375 is enriched in mouse islets compared with the other tissues, and miR-375 is released extracellularly following islets death. Indeed, increased plasma miR-375 have been observed in both streptozotocin-induced acute beta-cell death, and in NOD mice prior to diabetes onset [4]. Nevertheless, data in T1D subjects are largely inconclusive, with some reports showing increased circulating levels of miR-375 in patients with T1D, and others showing unchanged or decreased levels compared to control subjects without diabetes [4].

So far, all studies on miR-375 have been performed in very small cohorts of patients or in different settings, for example in cultured cells, body fluids, or solid tissue samples derived either from T1D patients or murine models of the disease [2, 4]. Consequently, findings are inconsistent among studies. Thus, studies in large and homogenous cohorts are warranted to test if miR-375 levels are altered in T1D subjects, and if miR-375 would be a useful biomarker of β-cell injury.

The aim of our study was to evaluate miR-375 levels in a very large and carefully selected cohort of 143 Sardinian subjects, which included subjects with T1D, first-degree relatives (FDR) of T1D patients, and healthy controls. We chose this particular population, because, in the incidence

Managed by Massimo Porta.

✉ M. G. Baroni
marco.baroni@uniroma1.it

[1] Endocrinology and Diabetes, Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

[2] Department of Medical Sciences and Public Health, University of Cagliari, Cagliari, Italy

of T1D, Sardinia is among the highest in the world, second only to Finland and three-to-four times higher than the other Italian regions, and it shows a strong familial aggregation [5].

Methods

Cohort characteristics are described elsewhere [5]. For this study, 49 T1D subjects, 46 autoantibodies (Aabs) positive FDR, and 48 controls were selected (Table 1). Follow-up data were available for 41 FDR, which resulted in 10/41 developing T1D, 8/41 impaired glucose regulation (IGR) (6 impaired fasting glucose and 2 impaired glucose tolerance), and 23/41 remained normo-glycaemic. GADA, IA-2A IAA, and ZnT8A measurements were performed as previously described [5].

For miRNA profiling, serum (200 µL) was added to 1 mL of QIAzol Lysis buffer and then spiked with 3.5 µL of miRNeasy Serum/Plasma Spike-In Control (Cel-miR-39, Quiagen). Total RNA was extracted using miRNeasy Serum/Plasma Kit (QIAGEN) and treated with DNase I. A reverse transcription of the miRNAs was performed with *TaqMan MicroRNA Reverse Transcription* kit, adding the specific primers of miRNA 375 (*TaqMan MicroRNA Assay hsa-miR-375) (Life Technologies)*. The cDNA was then amplified with *TaqMan Universal Master Mix II* and *TaqMan MicroRNA Assays hsa-miR-375* following the manufacturer's protocol. All samples were analyzed in duplicate and the Ct (cycle threshold) values were determined automatically by the analysis software.

Statistical analyses were performed with SPSS 20.0 statistical package. Differences between continuous variables across independent groups were evaluated by ANOVA for trend test. Correlations between continuous variables were calculated by Pearson's coefficient.

Results

In the overall population, we observed that the Aabs-positive FDR subjects have significantly increased levels of miR-375 compared to T1D patients and controls (Fig. 1). In particular, in Aabs-positive FDR subjects, miR-375 levels were increased 18-fold compared to controls and 15-fold compared to T1D patients (n. of copies: $5.15 \times 10^5 \pm 638{,}461$ vs $0.35 \times 10^5 \pm 91{,}676$ vs $0.26 \times 10^5 \pm 33{,}989$, respectively, p value $= 1 \times 10^{-6}$ after correction for multiple comparisons).

Within the Aabs-positive FDR subgroup, no association was found between the number of Aabs and miR-375 levels. However, in T1D patients and Aabs-positive FDR, miR-375 levels significantly correlated with higher c-peptide levels (correlation coefficient = 0.32, p value = 0.015).

Table 1 Clinical and biochemical parameters of study population

	T1D subjects ($n=49$)	Autoantibodies-positive first-degree relatives ($n=46$)[a]	Controls ($n=48$)
Sex (M/F)	30/19	17/29	20/28
Age (years)	16.1 ± 13	26.8 ± 18	41.2 ± 15
Weight (kg)	42.1 ± 19	–	–
BMI (kg/m^2)	19.8 ± 5	–	26.2 ± 5
DT1 duration (years)	4 ± 8	–	–
GADA (U/ml)	25.4 ± 44	18.1 ± 28	–
IA2 (U/ml)	5.8 ± 8	1.2 ± 2	–
IAA (U/ml)	9.1 ± 22	0.7 ± 1	–
Znt8 (U/ml)	229.7 ± 406	112 ± 265	–
BG 0′ (mg/dl)	381 ± 156	91 ± 8	92 ± 6
BG 120′ (mg/dl)	–	102 ± 33	98 ± 21
Ins 0′ (µUI/ml)	–	6 ± 3	–
Ins 120′ (µUI/ml)	–	22 ± 11	–
c-peptide (ng/ml)	0.8 ± 1	1.8 ± 0.9	–
miRNA (copies/ml)	$0.35 \times 10^5 \pm 91{,}676$	$5.15 \times 10^5 \pm 638{,}461$	$0.26 \times 10^5 \pm 33{,}989$

Values are expressed as means ± standard deviations or rate of subjects. P values < 0.05 are considered significant

BMI body mass index, *T1D* type 1 diabetes, *GADA* acid decarboxylase autoantibodies, *IA2* tyrosine phosphatase autoantibodies, *IAA* insulin autoantibodies, *ZnT8A* zinc transporter 8 autoantobodies, *BG* blood glucose, *Ins* insulin; glutamic

[a]At follow-up FDR subjects became: T1D = 10; IGR = 8 ; normo-glycaemic = 23. Follow-up data were not available for 5 FDR subjects

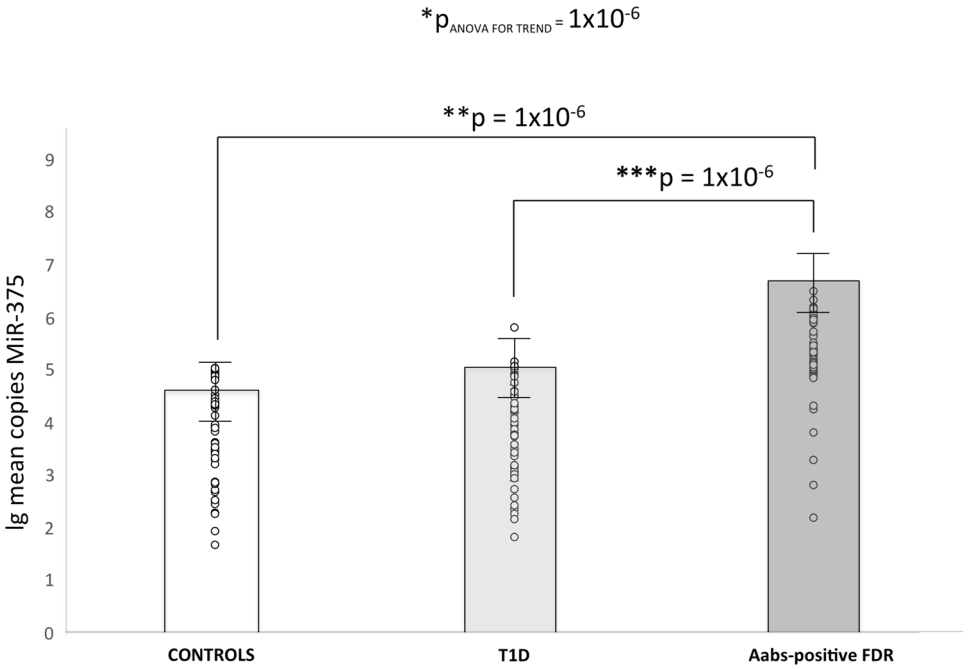

Fig. 1 Mir-375 levels in overall population stratified in type 1 diabetic (T1D) patients, autoantibodies-positive (Aabs-positive) first-degree relatives (FDR) of T1D patients and healthy controls. Mean MiR-375 copies were log-transformed because of the large scale of absolute number of copies. *P value (corrected for multiple comparisons) was calculated with ANOVA for trend test. p value $= 1 \times 10^{-6}$. **Comparison between controls and Aabs-positive subjects, p value $= 1 \times 10^{-6}$. ***Comparison between T1D patients and Aabs-positive subjects, p value $= 1 \times 10^{-6}$. The comparison between controls and T1D patients was not-significant

Furthermore, considering follow-up data, we observed that 10 Aabs-positive FDR subjects developed T1D and 8 subjects became IGR. Within these FDR subjects, those who developed T1D or IGR before 4 years (median of time of onset of diabetes and IGR in our population) had lower levels of miR-375 compared to FDR that developed T1D or IGR later (p value = 0.02).

We then constructed a ROC (receiver operating characteristic) curve to assess the possible predictive role of miR-375 levels on the diagnosis of T1D in the FDR that were followed longitudinally. MiR-375 levels did not show any predictive role, with an AUC (area under the curve) of 0.50 on T1D diagnosis. Instead, T1D autoantibodies were, as expected, highly predictive (AUC = 0.80).

Discussion

In conclusion, we observed that Aabs-positive FDR subjects showed significantly increased levels of miR-375 compared to T1D patients and controls. Moreover, we observed that increased circulating miR-375 associated with later onset of diabetes, suggesting a greater β-cell residual function. Interestingly, another study showed that miR-375 levels were lower in children with newly diagnosed T1D compared to age-matched control individuals [2]. These data suggest that circulating miR-375 levels may reflect the amount of viable beta-cells that are under autoimmune attack; thus, the closer to diagnosis, the lower miR-375 levels are. In addition, in agreement with this observation, the direct correlation between c-peptide levels and miR-375 levels could reflect higher β-cell residual function.

Hence, our results confirm the role of miR-375 in the autoimmune process of T1D, but its utility as a single biomarker does not emerge as clinically applicable.

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Author contributions LB, FS, and MGB conceived and designed the study. LB, MI, and FS conducted the experiments. EC recruited the study population. LB, FS, FAC, DB, and IB analyzed and interpreted the data. LB, FS, and MGB wrote the manuscript. MGB, MGC, and EC revised critically and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interests relevant to this study.

Ethical approval The study was approved by the Ethical Committee of the University of Cagliari and conducted in conformance with the Helsinki Declaration.

Informed consent Written consent was obtained from all the adult patients before the study. For children, written consent was obtained from the next of kin on behalf of the minors/children.

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